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13. Abstract *(Maximum 200 Words) (abstract should contain no proprietary or confidential information)*
Systemic chemotherapy including high dose chemotherapy with stem cell rescue frequently induces responses in women with metastatic breast cancer. Unfortunately, disease often recurs due to the persistence of chemotherapy resistant tumor cells. The immune system can effectively target and kill chemotherapy resistant tumor cells. Tumor associated antigens particularly antigens derived from the Her-2/neu oncogene can be recognized in breast cancer. Strategies to enhance immune recognition of these antigens may provide a therapeutic benefit. Recent studies indicate that the N-terminal flanking region of the invariant chain peptide termed CLIP has superagonisitc properties. The central hypothesis of this research project is that the N-terminal flanking region of CLIP can augment the immunogenicity of cryptic "self" peptide epitopes from Her-2/neu. Ongoing studies indicate that immunization with chimeric constructs of an MHC class II binding peptide antigen from Her-2/neu presented on tumor cells or on dendritic cells in concert with an MHC class I binding peptide from Her-2/neu induces a potent cytolytic T cell response and protective antitumor immunity. Further analysis of the cell-mediated immune mechanisms reveals that immunization with the chimeric Her-2/neu construct triggers a type 1 cytokine (IL-2, IFNγ) T helper cell response. The type 1 cytokine response may underlie the induction of protective antitumor immunity allowing for the clonal amplification of effector T cells and inducing upregulation of target antigens on the tumor cell. Importantly, immunization with the chimeric construct appears to enhance antitumor immunity even in the presence of growing tumors. Augmenting the immune response to tumor associated antigens will enhance therapeutic strategies for the treatment of breast cancer.

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1. Introduction:

Breast cancer is an increasingly common malignancy representing 30% of all cancers in women. Although conventional systemic chemotherapy or high dose chemotherapy with autologous stem cell rescue can induce remissions in a significant number of patients, complete curative responses are uncommon and nearly all patients die of progressive disease within 3 years.[1-6] Tumor recurrence and progression are thought to be due to the persistence of clones resistant to chemotherapy. Therefore, strategies that target these chemotherapy resistant tumor cells are required to have a significant impact on the treatment of breast cancer. Recent studies suggest that immunologic approaches may have meaningful clinical impact targeting chemotherapy resistant tumor cells.[7] In this regard, development of strategies to maximize antitumor immunity require augmenting immune mechanisms that specifically recognize tumor-associated antigens.[8,9,10] Recent studies suggest that the Her-2/neu oncogene can act as a tumor-associated antigen in breast cancer.[11,12] The development of vaccine strategies for breast cancer have focused around the findings that there appears to be active immunity to the Her-2/neu protein.[13,14] Unfortunately, the immune response is weak and ineffective largely due to the fact that Her-2/neu is a "self" protein with central tolerance to the immunodominant epitopes leaving only cryptic epitopes to be functionally recognized.[15] The principal focus of the current research project is to augment the immunogenicity of the cryptic epitopes from Her-2/neu inducing significant antitumor immunity. The central strategy of the proposed work is to strategically modify the cryptic epitope peptides from Her-2/neu with an amino acid sequence from the N-terminal flanking region of the invariant chain peptide termed CLIP. Previous studies have demonstrated theat the N-terminal flanking region of CLIP has superagonistic properties interacting with the Vβ segment of the T cell receptor increasing the affinity of the MHC class II-peptide-Tcell receptor complex.[16,17] Increasing the affinity of this complex leads to activation of the T lymphocytes capable of recognizing the specific peptide sequence. Thus, the current research proposal seeks to determine whether the immunogenicity of the Her-2/neu peptides can be augmented by the addition of the N-terminal region of CLIP, to characterize the immune response that is induced by immunization with the Her-2/neu chimeric peptide constructs and to determine whether immunization with these peptides elicits a heightened immune response leading to the induction of protective antitumor immunity.

Body:

Studies conducted during the first year of this proposal demonstrated that the amino acid sequence -KPVSP(M)- from the N-terminal flanking region could augment the immunogenicity of the p1171-1185 peptide (sequence –TLERPKTLSPGKNGV-) from Her-2/neu when presented as a chimeric construct to the immune system utilizing a c-neu positive rat breast cancer model. The initial studies also revealed that immunization with the chimeric Her-2/neu construct (either loaded onto irradiated tumor cells or presented on irradiated dendritic cells in concert with a weakly immunogenic MHC class I binding peptide from Her-2/neu) elicits potent cytolytic T cell responses to unmodified tumor cells. Moreover, immunization with the chimeric peptide construct induced protective antitumor immunity that required both CD4+ and CD8+ T lymphocytes as shown in adoptive transfer studies.

During the second year of this proposal, one of the major goals was to characterize the immune response elicited by immunization of animals with the chimeric Her-2/neu peptide construct. Initial studies revealed that there was no detectable antibody response (analysis of sera from 12 animals by enzyme-linked immunosorbent assay – ELISA) to either of the peptide regardless of immunization route (loaded tumor cells, loaded dendritic cells). Subsequent

studies focused on the cell-mediated immune response particularly the cytokine profile. Specific studies undertaken attempted to determine whether immunization with the chimeric Her-2/neu construct resulted in a heightened immune response to the parent, unmodified peptide. Animals were immunized peptide (parent p1171-1185 or the chimeric construct) loaded, irradiated (5000R) tumor cells (4 sites, 2.5 X 10^5 cells/site). Spleen cells were harvested and stimulated with antigen presenting cells loaded with the parent Her-2/neu peptide. The cells were analyzed by qualitative RT-PCR for type 1 (IL-2, IFNγ) and type 2 (IL-4, IL-10) cytokines. As illustrated in Figure 1, mRNA transcripts for both type 1 and type 2 cytokines were detected in the cultures of splenic T cells from the parental peptide immunized animals stimulated in vitro with the parent Her-2/neu peptide. In contrast, type 1 cytokine mRNA transcripts were preferentially detected in the cultures of chimeric peptide immunized animals stimulated with the parent peptide. In accord are the results from quantitative real-time RT-PCR (Figure 2) demonstrating pronounced levels of IFNγ mRNA transcripts after in vitro stimulation of the spleen cells from chimeric peptide immunized animals with the parental Her-2/neu peptide. Comparatively, mRNA transcripts for the type 2 cytokine IL-10 were markedly lower.

The frequency of responding T cells (largely CD4+ T lymphocytes established by flow cytometric analysis of T cell clones) were also assessed by limiting dilution. As shown in Figure 3, the frequency of responding T cells in animals immunized with the chimeric Her-2/neu construct responsive to the parental peptide was significantly increased compared to animals immunized with the parent peptide. Clonal analysis revealed an almost equal frequency of type 1 and type 2 producing cells (7clones with a type 1 profile; 8 clones with type 2 cytokine mRNA transcripts) from the parental peptide immunized animals in response to the unmodified, parent peptide. Comparatively, type1 cytokine producing cells were principally detected (14 clones producing type 1 cytokines; 2 clones producing type 2 cytokines) from chimeric peptide immunized animals stimulated in vitro with the parent Her-2/neu peptide. Normal rats not challenged with either peptide did not mount a detectable in vitro response to the parent Her-2/neu peptide.

Taken together, the results from our studies suggest that immunization of animals with the chimeric construct elicits a potent immune response to the parent, unmodified Her-2/neu peptide with a remarkable skewing to type 1 cytokine producing T cells. This skewing of the response appears to underlie the induction of antitumor immunity by immunization with the chimeric peptides providing sufficient IL-2 to clonally amplify cytolytic T cells. The production of IFNγ may also induce the upregulation of target antigens (MHC, Her-2/neu) on the tumor cells potentiating tumor cell recognition by the immune system. On the other hand, immunization with the parent Her-2/neu peptide elicits only a feeble immune response of both type 1 and type 2 cytokine producing T cells. As previously shown, this feeble immune response fails to provide protective antitumor immunity. In this setting, the induction of a type 2 cytokine may be immunoregulatory and negate the development of any antitumor immunity.

Ongoing studies are also evaluating the established clones for Vβ T cell receptor determinant expression (as detected by RT-PCR) to determine whether the repertoire is skewed, a potential outcome of immunization with the chimeric construct as based on our original hypothesis. To further evaluate the repertoire and to identify antigen specific responding T cells ex vivo, studies were initiated with a soluble, dimeric rat MHC class II – immunoglobulin (MHC class II-Ig) fusion protein (developed with institutional funds; the

molecular structure schematically illustrated in Figure 4). The soluble MHC class II-Ig construct was loaded with the parent or the chimeric Her-2/neu peptide and splenic T cells reactive with either peptide isolated by panning. The isolated T cells were analyzed by Vβ spectratyping which assesses the size of the complementarity determining region 3 (CDR3, peptide binding domain) of the T cell receptor.[19] The results (Figure 5) reveal that at least for Vβ8.5 positive T cells, there was an additional population of cells reactive to the chimeric construct compared to the parent Her-2/neu peptide. Planned studies include a full Vβ analysis of the isolated T cells and the analysis of cytokine mRNA transcripts by real-time RT-PCR.

Additional studies conducted during the current year evaluated whether antitumor immunity could be induced by immunization with the chimeric Her-2/neu construct in animals with actively growing tumors. The underlying rationale for this series of experiments was that immunotherapeutic approaches may offer the greatest benefit during the period of minimal residual disease after high dose chemotherapy or after autologous bone marrow transplantation (ABMT). Therefore, animals were challenged with tumor (intraperitoneally; 3×10^5) following ABMT and subsequently immunized (1X) with dendritic cells (4 sites, 2.5×10^4 cells/site) loaded with both the MHC class I binding Her-2/neu peptide and the MHC class II binding chimeric Her-2/neu construct 3 days later. Previous studies suggest that immunization with dendritic cells loaded with this combination of peptides is the most effective strategy to induce protective antitumor immunity in normal animals. Animals immunized with the peptide loaded dendritic cells exhibited a median survival of 37 days and 25% long-term survival (>70 days) as illustrated in Figure 6. Comparatively, control animals all succumbed to tumor growth by day 24. The results suggest that significant antitumor immunity can be induced in animals with actively growing tumors even after a single immunization. However, additional strategies are obviously necessary to maximize antitumor immunity in these animals. Studies in year three plan to compare different immunization regimens (i.e. increased frequency, increased numbers of dendritic cells) to maximize the induction of antitumor immunity in animals with actively growing tumors. Interestingly, preliminary studies reveal that administration of Cyclosporine following ABMT may increase the pools of autoreactive T cells that can recognize the tumor and can be activated by immunization with the chimeric Her-2/neu construct.

Key Research Accomplishments:

- demonstrated that immunization with the chimeric Her-2/neu construct elicits a potent cell-mediated immune response to the parent Her-2/neu peptide
- demonstrated that this immune response is skewed to T cells producing type 1 (IL-2, IFNγ) cytokines
- demonstrated that immunization with the parent peptide elicits only a feeble immune response with type 1 and type 2 cytokine producing T cells
- demonstrated that antitumor immunity can be induced in animals with actively growing tumors by immunization with the Her-2/neu chimeric construct

Reportable Outcomes:

The manuscript entitled "The N-terminal flanking region of the invariant chain peptide augments the immunogenicity of a cryptic 'self' epitope from a tumor-associated antigen" was accepted for publication in Clinical Immunology. This manuscript (galley proof appended) details the initial studies evaluating the ability of the chimeric Her-2/neu construct to induce protective antitumor immunity and the characterization of the immune response.

An abstract (appended) entitled "Augmenting antitumor immunity after autologous bone marrow transplantation: the impact of Cyclosporine on immunization" was submitted for the upcoming American Society of Hematology meeting. The abstract details the studies immunizing animals with the chimeric construct after tumor challenge. The abstract was accepted for presentation.

Conclusions:

The present project is based on the hypothesis that the N-terminal flanking region of the invariant chain peptide termed CLIP has superagonistic properties (by interacting with the Vβ chain of the T cell receptor) and can augment the immunogenicity of peptides from tumor-associated antigens. Previous studies indicate that the immunogenicity of a cryptic "self" epitope from the Her-2/neu tumor associated antigen can be augmented by immunization with chimeric peptide constructs containing the N-terminal flanking region of CLIP. This strategy elicits protective antitumor immunity. Current studies evaluated and characterized the cell-mediated immune response induced by immunization with the chimeric Her-2/neu peptide construct. The principal conclusion from these studies is that immunization with this chimeric construct induces a potent immune response. Most importantly, this response is highly skewed to cells producing type 1 cytokines that include IL-2 and IFNγ and may underlie the induction of protective antitumor immunity. Sufficient IL-2 may serve to amplify cytolytic effector mechanisms while IFNγ may serve to upregulate antigens on the tumor cell allowing effective immune recognition. On the other hand, immunization with the parent peptide only elicited a feeble response that included cells with type 1 and type 2 cytokine mRNA transcripts. The activation of the type 2 cytokine producing cells usually associated with immunoregulatory function may dampen any effective antitumor response. Additionally, the results from the current studies indicate that this approach (immunization with the chimeric Her-2/neu construct) can enhance the antitumor immune response in animals with actively growing tumors. Studies for the upcoming year plan to further evalute the function and repertoire of the responding T cells and to evaluate different strategies to enhance antitumor immunity in animals with actively growing tumors.

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Figure 1. Qualitative RT-PCR analysis of cytokine mRNA transcripts. Splenic T cells were harvested from animals immunized with the chimeric or unmodified peptide and stimulated in vitro with the parent Her-2/neu peptide.

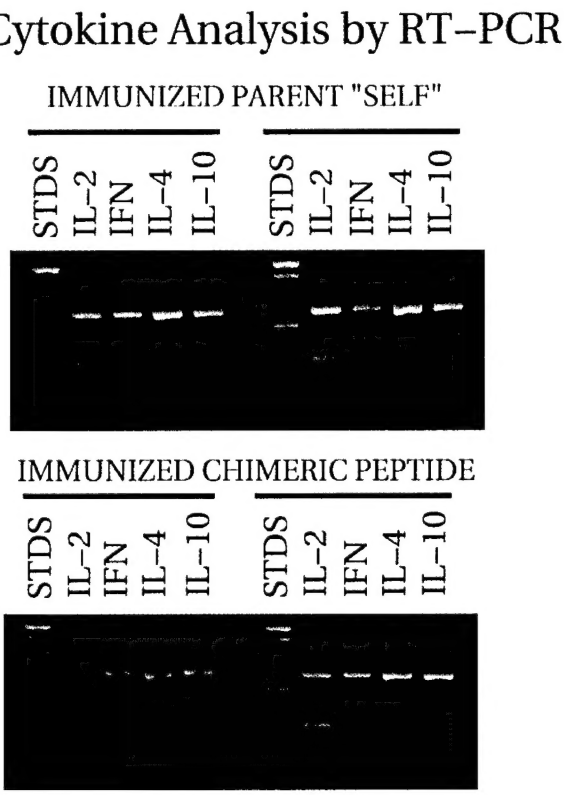


Figure 2. Real-time RT-PCR analysis of splenic T cells for cytokine mRNA transcripts. Animals were immunized with the chimeric or unmodified (parent) Her-2/neu peptide. Splenic T cells were harvested and stimulated in vitro with parental Her-2/neu peptide.

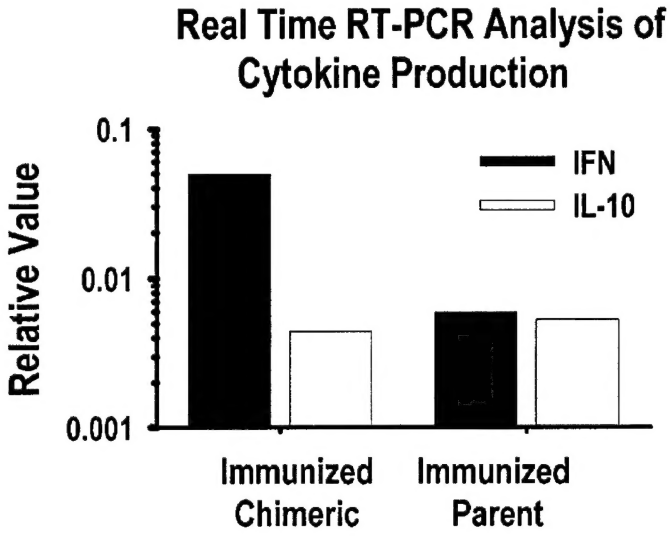
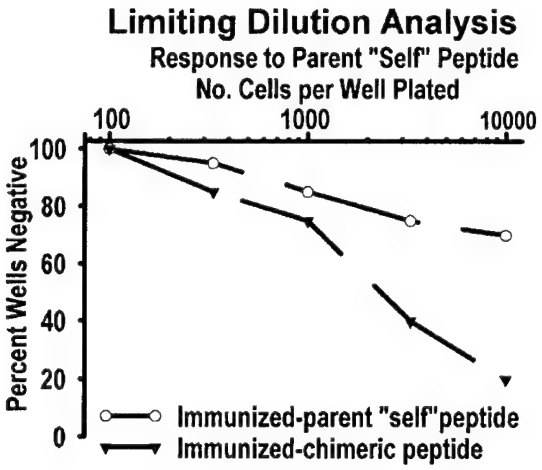


Figure 3. Limiting dilution analysis of the splenic T cell response to parental Her-2/neu peptide after immunization with either the chimeric construct or the unmodified peptide.



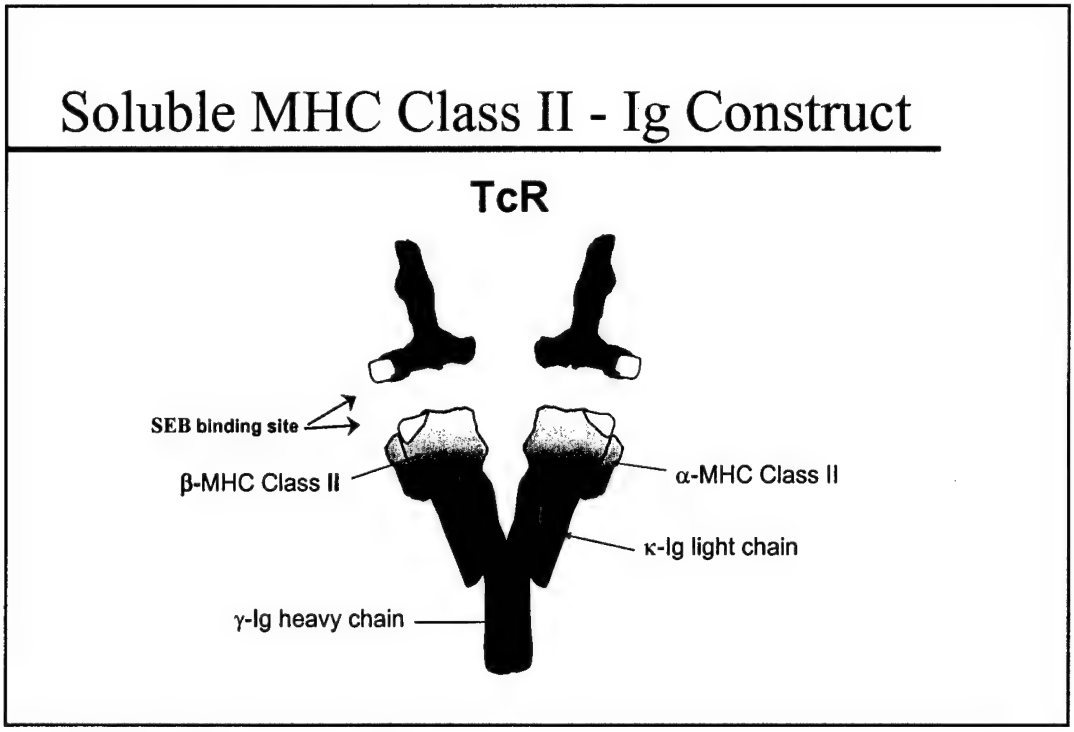


Figure 4. A dimeric soluble rat MHC class II molecule was molecularly constructed on a mouse immunoglobulin framework. The molecular construct was used to identify and isolate antigen-specific T cells by loading the MHC class II – Ig molecule with known peptide sequences.

Figure 5. Peripheral blood lymphocytes were selected for reactivity to the soluble MHC class II – Ig molecular construct loaded with either the unmodified Her-2/neu peptide or the chimeric construct by panning. The isolated cells were analyzed by CDR3 spectratyping for cells expressing the V-beta 8.5 T cell receptor determinant.

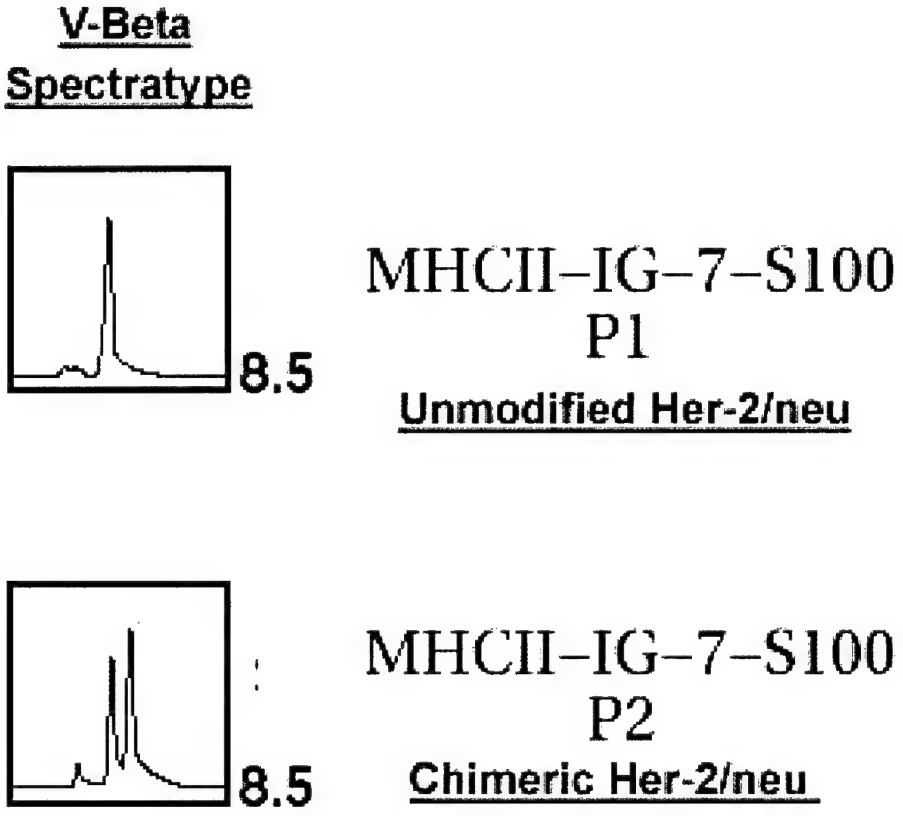
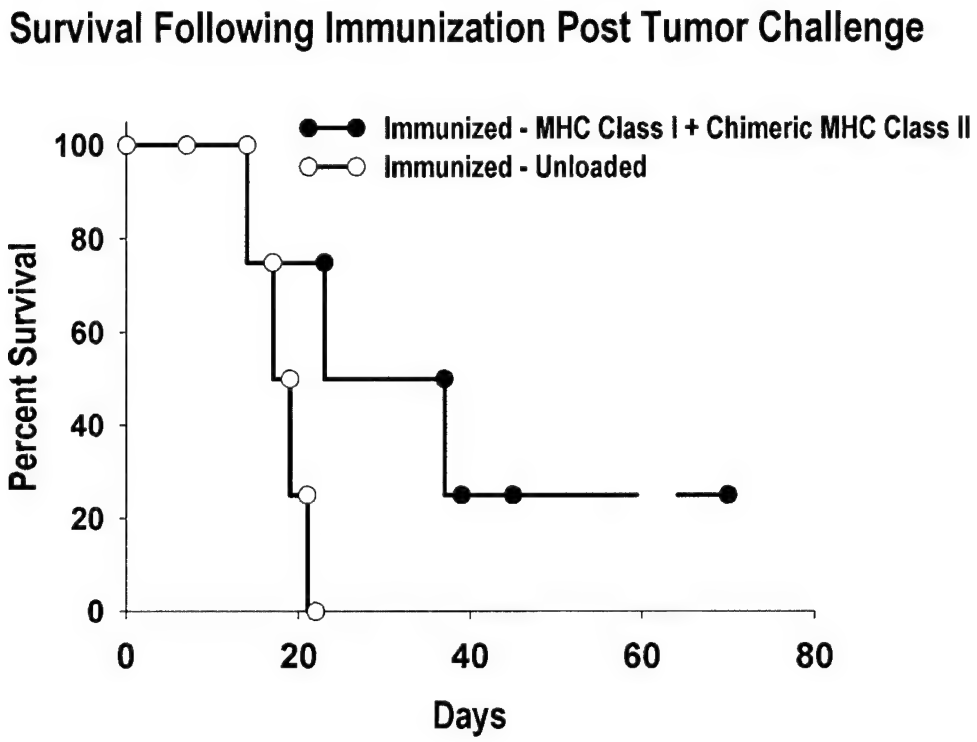


Figure 6. Animals received autologous (syngeneic) bone marrow transplants and challenged with tumor 3 X 10^5 intraperitoneally. Three days later, the animals were immunized with dendritic cells (2.5 X 10^4 cells/site X 4 sites) unloaded or loaded with the MHC class I Her-2/neu binding peptide and the chimeric MHC class II Her-2/neu construct. Normal animals not transplanted and not immunized succumbed to tumor growth within 20 days (data not included in graph).



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Augmenting Antitumor Immunity after Autologous Bone Marrow Transplantation: The Impact of Cyclosporine on Immunization

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Autologous bone marrow transplantation (ABMT) is an effective strategy for the treatment of lymphohematopoietic malignancies and solid tumors. Unfortunately, the rate of tumor recurrence is unacceptably high requiring the development of novel strategies including immunotherapy to augment the efficacy of ABMT. The present studies utilized a rat mammary cancer (CRL 1666) model to evaluate the development of antitumor immunity following BMT. For these studies, the strategy was to immunize F344 strain rats post ABMT with syngeneic dendritic cells (DC) pulsed with a limited set of peptides expressed by the tumor cells (one MHC class I:p554-562 and one MHC class II:p1171-1185 binding peptide from the c-erb oncogene). Without immunization, these animals succumb to tumor challenge (3 X 10^5,ip) within 14-18 days. Previous studies found that modifying the MHC class II binding peptide with the N-terminal flanking region (seq.-KPVSP-) of the invariant chain peptide, systemic

immunologic responses to the unmodified peptide could be significantly enhanced and preferentially evokes a type 1 cytokine response. Immunization of normal animals with the chimeric peptide prior (10d) to tumor challenge leads to protective immunity (survival > 70d) To explore potential mechanisms involved, the lymphocyte compartments in animals challenged with tumor before or after immunization were selectively modified by a short course of Cyclosporine (CsA;10mg/kg X 7d). This drug which inhibits thymic dependent clonal deletion may increase the pool of autoreactive T cells capable of responding to tumor. Immunization with peptide loaded DC post ABMT (day 7) prior to tumor challenge (day 14) elicited potent protective antitumor immunity with the animals resistant to tumor challenge. Immunotherapeutic approaches offer the greatest benefit during the period of minimal residual disease after ABMT. Therefore, animals were challenged with tumor following ABMT and subsequently immunized with peptide loaded DC 3 days later. Significant antitumor activity was induced with a median survival of 37 days and 25% long-term survival (>70 days). Comparatively, control animals (immunized with unloaded DC not receiving CsA or not transplanted) all succumbed tumor growth by day 24. Interestingly, tumor dose response studies in both experimental groups (immunization pre- and post tumor challenge) reveal that CsA treatment prior to immunization greatly enhances the generation of antitumor immunity. The results from the current studies indicate that immunization following ABMT enhances the induction of antitumor immunity. Although counter-intuitive,administration of CsA, by virtue of its ability to allow for the emergence of autoreactive T cells, may accentuate the induction of antitumor immunity by immunization after ABMT.

Disclosure Statement:
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The N-Terminal Flanking Region of the Invariant Chain Peptide Augments the Immunogenicity of a Cryptic "Self" Epitope from a Tumor-Associated Antigen[1]

Fn1 Fn2 Allan D. Hess, Christopher Thoburn, Weiran Chen, Yuji Miura, and Elsken Van der Wall[2]

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The N-terminal flanking region of the invariant chain peptide termed CLIP appears to have superagonistic properties interacting with the T cell receptor and the MHC class II molecule at or near the binding site for the bacterial superantigen Staphylococcal enterotoxin B (SEB). The present studies explored the hypothesis that the N-terminal segment of CLIP can augment the immunogenicity of cryptic "self" tumor-associated antigens. A chimeric construct of an MHC class II binding peptide from the c-erb oncogene (Her-2/neu) containing the N-terminal flanking region of CLIP elicited potent antitumor activity against a Her-2/neu-positive tumor in a rat model system. Comparatively, the unmodified (print) peptide was ineffective. The induction of effective antitumor immunity, however, required presentation of the chimeric peptide construct on irradiated tumor cells or the peptide construct in concert with a Her-2/neu MHC class I-restricted peptide from Her-2/neu. As revealed by adoptive transfer studies, effective protective antitumor immunity in this setting required the CD4 T helper subset. Additionally, *in vitro* analysis revealed that immunization with the parent peptide resulted in a weak immune response to the unmodified peptide consisting of both type 1 (IL-2, IFN-γ) and type 2 (IL-4, IL-10) cytokine-producing cells analyzed by RT-PCR (qualitative and quantitative) and by limiting dilution assay. Comparatively, immunization with the chimeric construct elicited a potent immune response to the parent peptide with predominantly type 1 cytokine-producing cells. Taken together, the results suggest that immunization with the chimeric Her-2/neu peptide induced protective antitumor immunity. Associated with this immunization strategy was the enhancement of a type 1 cytokine response.

PARENT

Key Words: **tumor vaccine; invariant chain peptide; modified Her-2/neu peptide.**

INTRODUCTION

During the past several years, evidence has accumulated indicating that tumor cells express antigens that can be recognized by the immune system (1–3). These tumor-associated (TA) antigens include normal "self" proteins that are overexpressed due to gene amplification (3, 4); however, the immune response to these antigens is weak and ineffective (5, 6). The host is tolerant to the immunodominant epitopes of these antigens, leaving only cryptic epitopes to be functionally recognized (7). The weak immune response to the cryptic epitopes of tumor antigens appears to be related to the low affinity of the peptide antigens for their presenting MHC molecule, resulting in poor presentation of the MHC–peptide ligands to T cells (8). In addition, T cells capable of responding to these antigens have clonotypic receptors with insufficient affinity for the peptide–MHC complex (7, 8). Augmenting the immunogenicity of these TA antigens is a critical step to developing vaccine strategies capable of eliciting effective antitumor immunity.

Characterization of the effector T cells in the experimentally induced autoaggression syndrome, termed autologous/syngeneic graft-vs-host disease (GVHD), reveals a unique mode of antigen recognition that augments recognition of nominal antigenic peptides (9). This autoaggression syndrome can be induced in man and in rodents by administering cyclosporine after autologous or syngeneic bone marrow transplantation and is associated with the development of a highly restricted repertoire of autoreactive T cells that promiscuously recognize MHC class II determinants (10–12). Recent studies reveal that the effector T cells recognize a peptide from the MHC class II invariant chain, termed CLIP, presented in the context of MHC class II antigens (10–14). There also appears to be a functional interaction between the Vβ component of

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the T cell receptor (TcR) and the N-terminal flanking region of the invariant chain peptide termed CLIP that extends beyond the peptide-binding domain of MHC class II. This superagonistic interaction that occurs at or near the binding site for the staphylococcal enterotoxin B (SEB) superantigen appears to increase the affinity of the TcR:MHC class II:peptide complex (10–14).

The present studies explore the hypothesis that the N-terminal flanking region of CLIP can augment the immunogenicity of cryptic "self" TA antigens. The results reveal that immunization of animals with a chimeric construct of a weakly immunogenic, MHC class II-restricted epitope from the c-erb (Her-2/neu) oncogene and the N-terminal flanking region of CLIP induced protective antitumor immunity. The induction of effective antitumor immunity, however, requires either presentation of the chimeric peptide construct on irradiated tumor cells or the peptide construct in concert with an MHC class I binding peptide from Her-2/neu presented on dendritic cells. Moreover, this immunization strategy elicited a dominant type 1 cytokine response.

MATERIALS AND METHODS

Animals

Fischer (F344) strain rats, 4–6 weeks of age, were purchased from Charles River, Inc. (Wilmington, MA). The animals were kept in sterile microisolator cages and fed food and water *ad libitum*. The animals were challenged with tumor intraperitoneally. For the adoptive transfer studies, the rats were pretreated with cyclophosphamide (100 mg/kg) 1 day prior to receiving immune spleen cells and tumor challenge. Four to six animals were used for each group.

Tumor Cells

The mammary adenocarcinoma cancer cell line CRL 1666, derived from F344 strain rats, was purchased from the American Type Culture Collection (ATCC, Rockville, MD). The tumor cells express MHC class I and II antigens and express c-erb (c-neu), as detected by mouse anti-rat c-neu monoclonal antibody (Ab-4; Oncogene Research Products, Calbiochem, Cambridge, MA). The cell line was maintained *in vitro* in McCoy's 5A tissue culture medium (Grand Island Biological Co., GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum. The cells were washed three times in tissue culture prior to use in *in vitro* assays or *in vivo* intraperitoneal challenge.

Effector Cell Isolation

Spleens from control and experimental animals were harvested and passed through a wire mesh screen to obtain a single cell suspension. The mononuclear cell fraction was isolated by Ficoll–Hypaque density centrifugation and further fractionated by nylon wool columns to enrich for T lymphocytes as previously described (15–18). $CD8^+$ and $CD4^+$ T lymphocyte subsets were isolated by immunomagnetic bead separation using the anti-rat CD4 and CD8 murine monoclonal antibodies (Serotec, Bioproducts for Science, Indianapolis, IN) as described previously (15). The purity of the population was confirmed flow cytometrically by staining the cells with monoclonal antibodies to rat CD4 and CD8 cell surface determinants and counterstaining with rat adsorbed, fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG (Sigma Chemical Co., St. Louis, MO). Cells stained with normal mouse serum and counterstained with the FITC anti-mouse IgG served as the control.

Dendritic cells were isolated from spleen cells based on differential plastic adherence, as previously described (16). Briefly, rat spleen cells were incubated for 2 h in tissue culture flasks. The flasks were rinsed thoroughly with tissue culture medium. After 18 h of incubation, the dendritic cells that detached from the plastic tissue culture flasks were harvested and washed in tissue culture medium. The cells were confirmed to be dendritic cells by their potent stimulatory activity of allogeneic lymphocytes in mixed lymphocyte reactions and by expression of OX62 (Pharmingen, San Diego, CA), the rat dendritic cell marker, assessed flow cytometrically.

Immunological Assessment

Killing was assessed using a [^{3}H]thymidine-based assay (JAM), as described by Matzinger, that measures DNA fragmentation and cell death (17). The target cells [phytohemagglutinin (PHA) blast cells, tumor cells; $5–10 \times 10^6$] were pulsed with 2.5 μCi/ml of [^{3}H]thymidine for 18 h and washed three times before assay. Graded numbers of the effector T cells and the target cells (5×10^3) were coincubated for 4 h before harvest. AQ: 1

The frequency of the responding T cells after vaccination was assessed utilizing a limiting dilution technique as previously described (12–15). Briefly, splenic lymphocytes were cultured at a limiting dilution utilizing irradiated syngeneic spleen cells loaded with parent or chimeric Her-2/neu peptides (MHC class II binding) as antigen-presenting cells in complete tissue culture medium containing IL-2 (10 U/ml). Positive wells were visually scored after 14 days of culture, and the clones were expanded by restimulation (every 7 to 10 days) with irradiated peptide-pulsed syngeneic spleen cells (2×10^4 cells/macrotiter well). Bulk cultures as well as T cell clones established form the limiting dilution cultures were also evaluated for cyto-

kine production by qualitative and quantitative RT–PCR (Taqman, Applied Biosystems, Foster City, CA), as previously described (13, 18). Real-time PCR reactions were performed using the Taqman assay (Applied Biosystems) with fluorescent primers, as previously described (18). Data were analyzed with Sequencer Detection version 1.6 software. The threshold cycle during the exponential phase of amplification was determined by real-time monitoring of fluorescent emission after cleavage of sequence-specific probes by nuclease activity of *Taq* polymerase. Results from quantitative RT–PCR were also normalized against mRNA transcripts for GADPH evaluated by real-time PCR.

Peptides

The sequences of the peptides principally utilized in the present studies are given in Table 1 and include the truncated variant of CLIP containing the N-terminal flanking region (p86–100), the fluoresceinated derivative of p86–100 (for binding studies), the parent MHC class II binding c-erb (Her-2/neu) peptide [p1171–1185; identical sequence to human Her-2/neu described by Dsis *et al.* (19, 20)], and the chimeric derivative containing the N-terminal flanking region of CLIP.

Recent studies have elucidated the binding motif for Lewis/F344 strain rats (21). The peptide ligands are nonamers that contain a hydrophobic leucine anchor residue at position 3 and a carboxyl-terminal serine anchor residue. Computer modeling of the rat Her-2/neu amino acid sequence revealed five potential candidates that could bind to Lewis/F344 MHC class I molecules. Initial studies revealed one sequence (p554–562) that elicits a cytolytic T cell response (described in the results and listed in Table 1). The other peptides (p377–385, p403–411, p439–447, p790–798, and p1105–1113) were either weakly or completely nonimmunogenic. The peptides, chemically synthesized and purified by high-pressure liquid chromatography, were obtained from Quality Controlled Biochemicals (Hopkinton, MA). The peptides (>92% purity) were diluted to 10 μM in RPMI 1640 prior to loading, as previously described (10, 11, 13). Previous dose-response studies revealed that maximal saturation was achieved by pretreating the target cells with 1 μM peptide.

TABLE 1

Peptide Sequences

Peptide	Sequence
MHC class II–parent Her-2/neu (p1171–1185)	TLERPKTLSPGKNGV
Chimeric Her-2/neu	KPVSPMTLERPKTLSPGKNGV
N-Terminal CLIP variant (p86–100)	KPVSPMRMATPLLMRS
MHC class I–Her-2/neu (p554–562)	KGLPREYVS

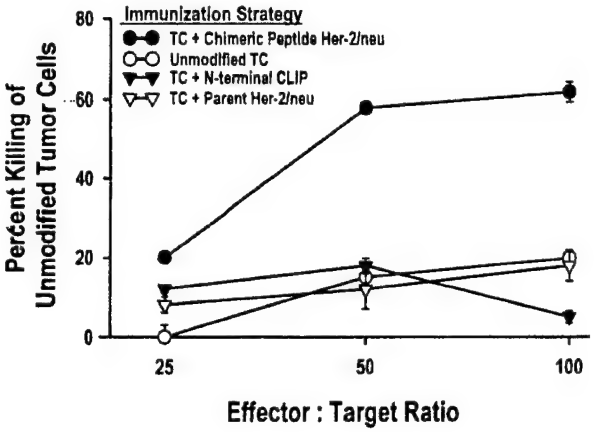

FIG. 1. Immunization with chimeric Her-2/neu peptide-loaded tumor cells (TC) induces a cytolytic T cell response. F344 rats were immunized intradermally (four sites, twice, 14 days apart) with irradiated (5000 R) CRL 1666 tumor cells (2.5×10^6) loaded with the p1171–1185 Her-2/neu peptide, the p1171–1185 chimeric construct, the truncated variant of CLIP containing the N-terminal flanking region, or the control diluent. Fourteen days following the last immunization, splenic T cells were harvested and assessed for their ability to kill *unmodified* CRL 1666 tumor cells.

RESULTS

Initial studies using a whole-cell immunization strategy evaluated whether the -KPVSP(M)- sequence from the N-terminal flanking region of CLIP could augment the immunogenicity of the p1171–1185 peptide from Her-2/neu. This peptide, as decribed in previous studies, is weakly immunogenic (19, 20). Tumor cells were loaded with the parent peptide or the chimeric construct. As a control, the tumor cells were loaded with the N-terminal truncated variant of CLIP or left unloaded. The loaded tumor cells were irradiated (5000 R) and injected subcutaneously at four sites (2.5×10^5 cells per site) on the back of the animals. The animals were reimmunized 14 days later. Two weeks following the second immunization, the animals were either evaluated immunologically or challenged with viable tumor cells administered intraperitoneally. A representative (1/4) experiment is presented in Fig. 1. Immunization of animals with tumor cells loaded with the chimeric peptide elicited a potent cytolytic T cell response capable of killing unmodified tumor cells (T cell identity confimed in depletion experiments—removal of cells expressing the α/β-T cell receptor removed lytic activity; data not shown). In comparison, immunization of animals with tumor cells loaded with

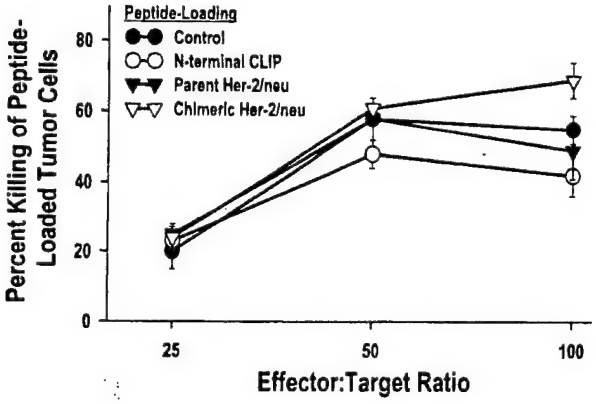

FIG. 2. Effect of peptide loading on tumor cell killing. Spleens from animals immunized twice with the p1171–1185 chimeric peptide-loaded tumor cells were harvested 14 days after the second immunization. The splenic lymphocytes were isolated by Ficoll–Hypaque density centrifugation and enriched for T cells by nylon wool fractionation. The effector T cells were assessed for their ability to kill tumor cells loaded with the p1171–1185 peptide from Her-2/neu, the p1171–1185 chimeric construct, the N-terminal truncated variant of CLIP, or the control diluent.

either the parent Her-2/neu or the N-terminal truncated variant of CLIP did not result in the induction of any significant cytolytic T cell activity against the unmodified tumor cells.

The specificity of the cytolytic response (in order to determine whether the chimeric MHC class II binding peptide was recognized by the cytolytic T cells or whether other antigens were now effectively recognized) was further explored by evaluating the ability of the cells from the animals immunized with the chimeric Her-2/neu peptide to kill peptide-loaded tumor cells. As shown in Fig. 2, loading the tumor cells with F2 the chimeric peptide or the other MHC class II binding peptides (unmodified parent Her-2/neu peptide, the N-terminal truncated CLIP variant) had little effect on the susceptibility of the target cells to killing mediated by the primed effector cells. Therefore, rather than a cytolytic response to the chimeric peptide, this whole-cell immunization strategy allowed for the development of lytic T cells capable of recognizing unmodified tumor cells.

The immune response (specifically to the parent, unmodified peptide) following immunization with the parental and chimeric peptide was also evaluated *in vitro*. Splenic T lymphocytes from the animals immunized with the parental or chimeric peptide were stimulated in bulk culture and analyzed by qualitative RT–PCR for type 1 and 2 cytokines. As shown in Fig. 3A, both type 1 (IL-2, IFN-γ) and type 2 (IL-4, IL-10) F3 mRNA transcripts were detected in the cultures of splenic T cells from the parental peptide-immunized animals stimulated with the parent peptide. In contrast, type 1 cytokine mRNA transcripts were preferentially detected in the culture of chimeric peptide-immunized animals stimulated with the parental peptide. In accord are the results from quantitative real-time RT–PCR (Fig. 3B), demonstrating pronounced levels of IFN-γ mRNA transcripts after *in vitro* stimulation with parental peptide of the spleen cells from chimeric peptide-immunized animals. Levels of mRNA transcripts for IL-4 were markedly lower. 10
The frequency of responding cells (largely $CD4^+$ T cells

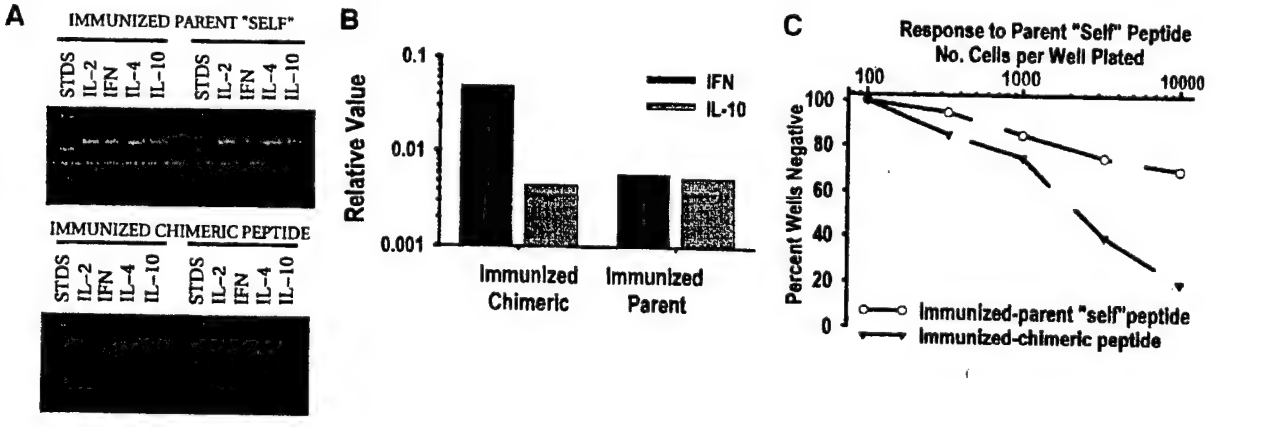

FIG. 3. *In vitro* analysis of lymphocyte responses following immunization. Animals were immunized with peptide-loaded, irradiated (5000 R) tumor cells (parent p1171–1185 and the chimeric construct). Splenic T cells were harvested and both bulk (2×10^6 cells/ml) and limiting dilution cultures established, stimulating with antigen-presenting cells (10^4, bulk; 10^3, limiting dilution) loaded with the parent Her-2/neu peptide. (A) Qualitative RT–PCR analysis for type 1 and type 2 cytokine mRNA transcripts. (B) Quantitative (real-time) RT–PCR analysis for IFN-γ and IL-10 mRNA transcripts (normalized against mRNA transcripts for GADPH). (C) Limiting dilution analysis.

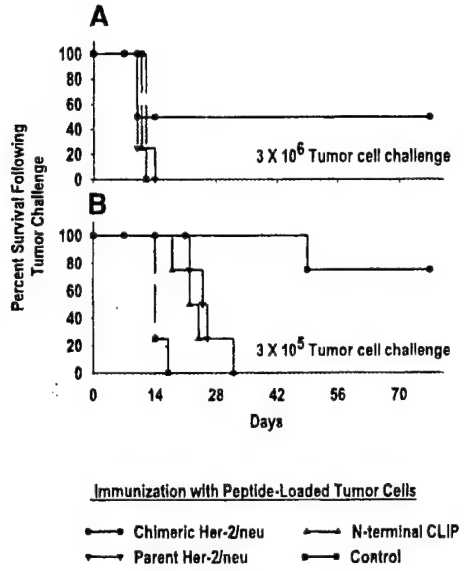

FIG. 4. Immunization with chimeric Her-2/neu peptide-loaded tumor cells induces protective antitumor immunity. F344 rats were immunized (twice, 14 days apart) with peptide-loaded, irradiated (5000 R) CRL 1666 tumor cells. Included in the panel of peptides were the parent p1171–1185 peptide, the chimeric construct, and the truncated variant of CLIP containing the N-terminal flanking region. Fourteen days following the second immunization, the animals were challenged with live tumor cells administered intraperitoneally.

established by flow cytometric analysis of T cell clones; data not presented) was also assessed in limiting dilution. As shown in Fig. 3C, the frequency of responding T cells in animals immunized with the chimeric construct responsive to the parental peptide was significantly increased compared to that in animals immunized with the parent peptide. Clonal analysis of the cytokine profile revealed an almost equal frequency of type 1- and 2-producing cells (7 of type 1; 8 of type 2) from the parental peptide immunized animals in response to stimulation with the unmodified peptide. Comparatively, type 1 cytokine-producing cells were principally detected (14 of type 1; 2 of type 2) from chimeric peptide-immunized animals followed by *in vitro* stimulation with the parental peptide. Normal rats not challenged with either peptide or tumor did not mount a detectable response to the parental peptide.

Immunization of the animals with the tumor cells loaded with the chimeric Her-2/neu peptide resulted in the induction of protective antitumor immunity. As F4 shown in Fig. 4, animals immunized with the chimeric Her-2/neu tumor cell preparation were resistant to live tumor cell challenge. Vaccination with the chimeric peptide resulted in 50% of the animals being resistant to challenge with 3×10^6 live tumor cells. Comparatively, animals immunized with the tumor cells loaded with the parent peptide or the N-terminal truncated variant of CLIP all succumbed to tumor growth by day 15. At a lower tumor cell challenge dose (3×10^5), 75% of the animals vaccinated with the chimeric Her-2/neu tumor cell preparation survived. There was, at best, only a marginal effect when the animals were immunized with the tumor cells loaded with the parent peptide or the N-terminal truncated variant of CLIP. Nevertheless, these animals succumbed to tumor challenge by day 32.

The efficacy of the chimeric Her-2/neu peptide construct was also evaluated utilizing peptide-loaded dendritic cells. These studies, however, would only be effective if an MHC class I-restricted peptide could be identified that elicited a cytolytic T cell response and was expressed on the tumor cells. Recent studies have identified the binding motif for MHC class I molecules in F344 rats (21). Based on computer modeling, six potential MHC class I binding peptide candidates were identified. One peptide (p554–562) was found to be immunogenic. As demonstrated in Fig. 5A, immuniza- F5 tion of animals with dendritic cells (5×10^4 cells per site, four sites) loaded with the MHC class I binding peptide elicited a cytolytic T cell response. Spleen cells from these animals were capable of killing PHA blast

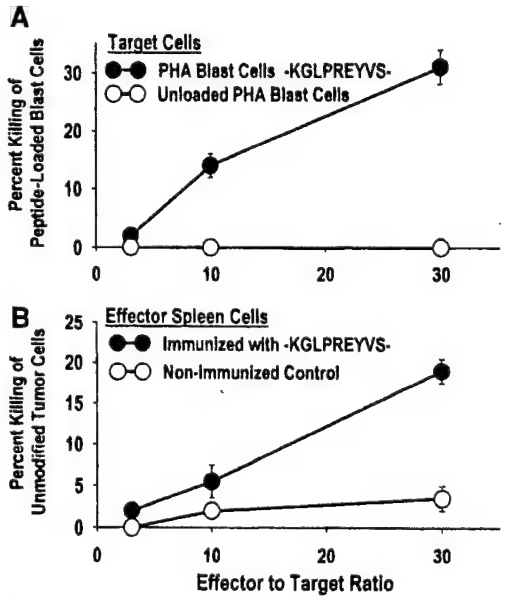

FIG. 5. Induction of a cytolytic T cell response to a Her-2/neu MHC class I-restricted peptide. F344 rats were immunized intradermally (twice, 14 days apart) with dendritic cells (5×10^4 cells/site, four sites) loaded with the MHC class I binding peptide (p554–562) from Her-2/neu. Splenic T lymphocytes were harvested 14 days later and evaluated for their ability to kill peptide-loaded PHA blast cells (A) or *unmodified* CRL 1666 tumor cells (B).

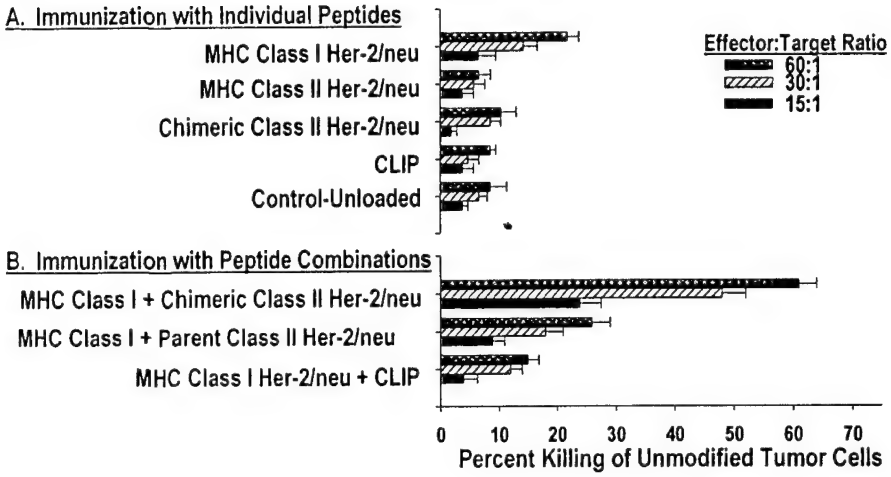

FIG. 6. *In vitro* targeting of unmodified tumor cells after immunization with Her-2/neu peptide-loaded dendritic cells. F344 rats were immunized intradermally twice, 14 days apart with peptide-loaded dendritic cells (5×10^4 cells/site, four sites). Peptides included the p1171–1185 parent, the chimeric construct, the N-terminal CLIP variant, and the MHC class I binding peptide (p544–562) from Her-2/neu. Peptides were loaded singly (A) or in various combinations (B). Subsequent (14 days) to the last immunization, splenic T lymphocytes were harvested and evaluated for their ability to kill *unmodified* CRL 1666 tumor cells.

cells loaded with this peptide, but demonstrated no specific killing of unloaded PHA blast cells. In addition, no specific killing of the target cells could be demonstrated if the nonimmunogenic peptides were loaded (data not shown). More importantly, as shown in Fig. 5B, spleen cells from the immunized animals were able to kill unmodified tumor cells, clearly indicating that this peptide is presented by this tumor cell line. These findings were confirmed in three additional animals immunized with the peptide-loaded dendritic cells.

Based on these findings, dendritic cells were loaded with combinations of the MHC class I binding peptide and the parent or the chimeric MHC class II binding peptides. Additionally, the dendritic cells were also loaded with the truncated variant of CLIP containing the N-terminal flanking region. Animals were immunized with the peptide-loaded dendritic cells (5×10^4 cells/site at four sites $\times$ 2; 14 days apart). Control animals were immunized with unloaded dendritic cells. Subsequent to the last immunization (14 days), the animals were evaluated for cytolytic T cell function and for the induction of protective antitumor immu- F6 nity. As shown in Fig. 6, a potent cytolytic T cell response could only be demonstrated in animals immunized with the dendritic cells loaded with the MHC class I Her-2/neu peptide and the chimeric MHC class II Her-2/neu construct (Fig. 6B). These cytolytic T cells belonged to the CD 8^+ T cell subset, as confirmed in depletion experiments (percentage killing at a 30:1 effector to target ratio: mean $\pm$ SEM, $n = 3$; control, 52.3 $\pm$ 4.9; CD4 depleted, 48.3 $\pm$ 3.7; CD8 depleted, 2.6 $\pm$ 2.8). Weak or modest cytolytic T cell responses could be demonstrated after immunization with dendritic cells loaded with the MHC class I Her-2/neu peptide plus the parent MHC class II Her-2/neu peptide (Fig. 6B) or with only the MHC class I peptide from Her-2/neu (Fig. 6A). In accord with these results are the findings that the animals immunized with the MHC class I peptide from Her-2/neu plus the chimeric construct presented on dendritic cells were resistant to live tumor challenge (Fig. 7B). Comparatively, all other F7 groups immunized with dendritic cells variably loaded with the different peptide combinations succumbed to tumor challenge (Figs. 7A and 7B).

Studies were undertaken to evaluate whether protective antitumor immunity required both $CD4^+$ and $CD8^+$ T cells. Spleen cells from the immunized animals and resistant to tumor challenge were harvested and fractionated into the $CD4^+$ and $CD8^+$ T cell subsets prior to adoptive transfer into naive F344 rats. The rats were challenged with 3×10^5 live tumor cells. As shown in Fig. 8, animals receiving unfractionated F8 spleen cells or the combination of $CD4^+$ and $CD8^+$ T lymphocyte subsets were resistant to tumor challenge. Animals receiving just the isolated $CD4^+$ or $CD8^+$ T cell subset succumbed to tumor challenge.

One potential reason that would account for the heightened immunogenicity of the chimeric peptide construct is that it might have an increased affinity for MHC class II molecules compared to the parent, unmodified peptide. Studies were undertaken to evaluate whether the chimeric peptide construct had a greater

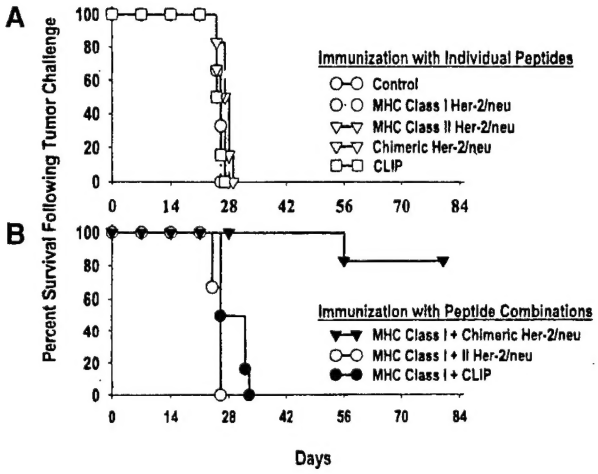

FIG. 7. Immunization with Her-2/neu peptide-loaded dendritic cells induces protective antitumor immunity. F344 rats were immunized intradermally (twice, 14 days apart) with dendritic cells (5×10^4 cells/site, four sites) that were loaded singly (A) or in various combinations (B) with the MHC class I (p554–562) and class II (p1175–1185) Her-2/neu peptides, the chimeric construct, or the N-terminal CLIP variant. Control animals received unloaded dendritic cells. Fourteen days later, the animals were challenged with live tumor (3×10^5 CRL 1666 cells) administered intraperitoneally.

affinity than the parent molecule. In order to approach this question, a flow cytometric assay was developed in which PHA blast cells were stained with fluoresceinated CLIP. As shown in Fig. 9, both the parent and the chimeric Her-2/neu construct equally inhibited the

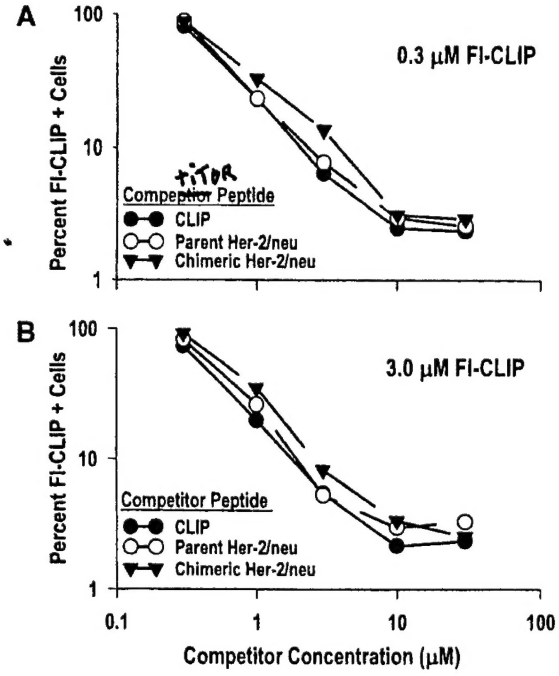

FIG. 9. Inhibition of FL-CLIP binding by parent and chimeric Her-2/neu peptides. PHA blast cells were incubated for 2 h at 4°C with fluoresceinated CLIP (A, 0.3 μM; B, 3.0 μM) in the presence of graded quantities of the parent p1171–1185 peptide, the chimeric construct, or the N-terminal variant of CLIP. The cells were washed prior to flow cytometric analysis on an EPICS IV Coulter flow cytometer (Coulter, Hialeah, FL) evaluating the percentage of cells staining with the fluoresceinated CLIP in the presence the different peptides.

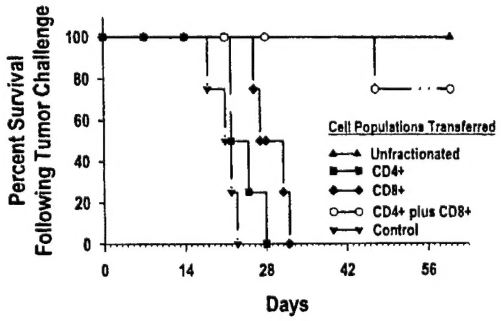

FIG. 8. Effective antitumor immunity requires both $CD4^+$ and $CD8^+$ T cells. Animals were immunized (twice) intradermally with dendritic cells (5×10^4 cells/site, four sites) loaded with the MHC class I (p554–562) and the chimeric (p1171–1185) construct. Fourteen days after the last immunization, the splenic T lymphocytes were harvested and the $CD8^+$ and $CD4^+$ subsets isolated by immunomagnetic bead separation. The cells were adoptively transferred into secondary F344 recipients (unfractionated, isolated $CD4^+$ and $CD8^+$ subsets; 30×10^6 cells per recipient; recombined subsets, 15×10^6 of each per recipient). Following (1 day) the adoptive transfer, the animals were challenged with 3×10^5 viable CRL1666 tumor cells.

binding of fluorescent CLIP. This ability to inhibit fluorescent CLIP binding was virtually identical to the ability of native CLIP to inhibit the binding of the fluoresceinated derivative. Previous studies confirmed binding of the fluoresceinated CLIP to MHC class II, since pretreatment of the PHA blast cells with monoclonal antibody to MHC class II determinants inhibited staining, whereas anti-MHC class I antibody pretreatment was ineffective (10, 12).

DISCUSSION

Previous studies in our laboratory revealed that the autoaggression syndrome induced by administering cyclosporine after syngeneic or autologous bone marrow transplantation is mediated by a highly conserved repertoire of $V\beta$ 8.5^+ $CD8^+$ autoreactive T cells that promiscuously recognize MHC class II determinants (8, 15). Recognition of MHC class II molecules by the autoreactive T cells is dependent on the presentation and recognition of CLIP (10, 12). This peptide derived

from the invariant chain that shepherds the biosynthesis of MHC class II is thought to stabilize MHC class II molecules in the absence of nominal peptides (22, 23). Essential for the promiscuous recognition of MHC class II, however, is the interaction between the N-terminal flanking region of CLIP that extends beyond the MHC peptide-binding groove and the Vβ segment of the TcR at or near the binding site for the superantigen SEB (12, 13, 24, 25). This interaction could overcome the requisite specificity of the TcR CDR3 domain for the peptide sequence within the peptide binding groove of MHC class II. Presentation of chimeric constructs of irrelevant peptides with the N-terminal flanking region allowed for effective targeting by CLIP-reactive T cell clones (13). Of additional importance in this regard are the findings that the N-terminal fragment of CLIP can promote promiscuous binding of peptides to MHC class II when presented as chimeric constructs (25). The affinity of the TcR:peptide:MHC class II complex appears to be increased by this interaction, thus potentially explaining not only the promiscuous specificity of the autoreactive T cells but also the restriction of the repertoire to an SEB-responsive subset. Moreover, this interaction appears to override the requirement for the classical cell surface accessory molecule (CD4) (11, 12).

The superagonistic properties of the N-terminal flanking region of CLIP as defined in the autologous/syngeneic GVHD model and its ability to promote promiscuous binding of peptides to MHC class II suggest that this peptide fragment may be able to augment the immunogenicity of nominal peptides, including cryptic "self" epitopes from TA antigens. The results from the present studies in a model system clearly support this hypothesis. The immunogenicity of a known MHC class II binding peptide (p1171–1185) from the rat (c-neu) Her-2/neu oncogene was augmented by the addition of the N-terminal flanking region sequence of CLIP. This peptide was weakly immunogenic, eliciting both antibody and CD4 T helper responses, but required repeated immunizations in adjuvant to evoke this response (19, 20). Immunization with this parent peptide also failed to induce significant protective antitumor immunity. In the present studies, immunization with a chimeric construct of this peptide, which contained the N-terminal flanking region of CLIP, elicited a potent cytolytic T cell response and the induction of protective antitumor immunity. Successful immunization required presentation of both the N-terminal flanking region and the Her-2/neu peptide. Interestingly, immunization with the chimeric construct increased the frequency of cells responding to the parent peptide and skewed the repertoire to type 1 cytokine-producing cells. In accord are recent findings demonstrating that the potency of MHC class II-presented epitopes is increased by linking it to the p77–92 peptide of the invariant chain (26). Moreover, studies by Naujokas *et al.* suggest that there is an interactive T cell epitope on a flanking region of CLIP that lies outside of the MHC class II peptide-binding domain (27).

It is important to note, however, that the induction of a successful antitumor response *in vitro* and *in vivo* required either presentation of the chimeric peptide on irradiated tumor cells or the peptide construct in combination with an MHC class I binding peptide from Her-2/neu presented on dendritic cells, whereas other combinations or the use of single peptides was ineffective. These data suggest that although the immunogenicity of the p1171–1185 chimeric construct was enhanced (also evidenced by the results from the limiting dilution studies), there was a requirement for direct recognition of the tumor cell (and presentation of other MHC class I-restricted antigens?) or presentation of a Her-2/neu antigen restricted by MHC class I and expressed on the tumor cell in combination with the chimeric construct. The chimeric construct did not appear to be recognized by the cytolytic T cells. These results were initially surprising, considering the findings in the autologous/syngeneic GVHD model in which the N-terminal flanking region of CLIP allowed for $CD8^+$ cytolytic T cell targeting of MHC class II (9–14). Perhaps this unique mode of MHC class II antigen recognition is dependent on the administration of cyclosporine and its affect on T cell differentiation in the thymus. A number of studies clearly indicate that this drug remarkably alters thymic differentiation and restriction (28–30). It will be of interest to evaluate the chimeric vaccine strategy after a course of cyclosporine treatment.

For peptide vaccine strategies to be successful, the tumor cells must express the antigen at the cell surface. In this regard, recent studies by Zaks and Rosenberg demonstrated that immunization with a peptide epitope from Her-2/neu elicited peptide-specific cytolytic T lymphocytes but failed to recognize Her-2/neu-positive tumors (31). In the present studies, there was recognition and killing of the unmodified tumor cells after immunization with the MHC class I binding peptide. This is not surprising, since established peptide-specific T cell clones can target and kill unmodified tumor cells, confirming that this peptide is expressed by the tumor cell. A potent cytolytic T cell response and the induction of protective antitumor immunity, however, required immunization with the MHC class I peptide in conjunction with the chimeric construct. The adoptive transfer studies clearly show that protective antitumor immunity required priming of the $CD4^+$ T cell subset, findings that are in accord with those of several other studies (32–36). It seems likely that immunization with the chimeric construct primed the $CD4^+$ T cell subset. The underlying mechanisms accounting for the heightened immunogenicity of the chi-

meric construct remain unclear. Competitive inhibition studies did not reveal any significant differences in affinity between the parent peptide and the chimeric construct that might account for the potentiation of immunogenicity (25, 26). The potential interaction between the N-terminal flanking region and the Vβ segment of the T cell receptor may account for the potentiation of nominal peptide immunogenicity (12, 13, 26). Our previous studies suggest that the interaction between the N-terminal flanking region of CLIP and the Vβ segment of the TcR as defined for the autologous/syngeneic GVHD effector T cells occurs at or near the SEB binding site. Such an interaction might skew the repertoire, and studies are ongoing to evaluate the V-region repertoire after immunization. The results from the present studies also suggest that superantigens, such as SEB, may be useful to augment antitumor immunity. One potential explanation for the induction of effective antitumor immunity in the present studies, however, is the skewing of the immune response with an increased frequency of type 1 cytokine-producing cells. The interaction of the N-terminal flanking region with the TcR may provide a sufficiently strong signal to facilitate a type 1 response (39). Comparatively, immunization with the parental peptide resulted in both type 1 and 2 cytokine-producing cells. The induction of an "equivalent" type 2 response that is usually associated with immunoregulation may negate the development of any effective antitumor immunity.

The results from the present studies utilizing a model system indicate that the N-terminal flanking region of CLIP can augment the immunogenicity of a cryptic epitope from a "self" TA antigen. The broad applicability of this system, however, will be dependent on the identification of MHC class II-dependent antigenic epitopes from TA antigens. Studies are also underway to evaluate other peptides constructs from Her-2/neu and to determine whether this approach can be effective in animals with actively growing tumors.

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